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Review Article

Flow cytometry-based diagnosis of primary immunodeficiency diseases

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Abbreviations:
ADA, adenosine deaminase; ALPS, autoimmune lymphoproliferative syndrome; APECED, autoimmune polyendocrinopathy-candidiasis–ectodermal dystrophy/dysplasia; BAFF-R, B-cell activating factor receptor; BTK, Burton's tyrosine kinase; CD40L, CD40 ligand; CGD, chronic granulomatous disease; CMCD, chronic mucocutaneous candidiasis; CTLA4, cytotoxic T-lymphocyte-associated protein 4; CTLs, cytotoxic T lymphocytes; CVID, common variable immunodeficiency; DHR, dihydrorhodamine; DNT, double-negative T; DOCK8, dedicator of cytokinesis

ABSTRACT

Primary immunodeficiencies (PIDs) are a heterogeneous group of inherited diseases of the immune system. The definite diagnosis of PID is ascertained by genetic analysis; however, this takes time and is costly. Flow cytometry provides a rapid and highly sensitive tool for diagnosis of PIDs.

Flow cytometry can evaluate specific cell populations and subpopulations, cell surface, intracellular and intranuclear proteins, biologic effects associated with specific immune defects, and certain functional immune characteristics, each being useful for the diagnosis and evaluation of PIDs. Flow cytometry effectively identifies major forms of PIDs, including severe combined immunodeficiency, X-linked agammaglobulinemia, hyper IgM syndromes, Wiskott-Aldrich syndrome, X-linked lymphoproliferative syndrome, familial hemophagocytic lymphohistiocytosis, autoimmune lymphoproliferative syndrome, IPEX syndrome, CTLA 4 haploinsufficiency and LRBA deficiency, IRAK4 and MyD88 deficiencies, Mendelian susceptibility to mycobacterial disease, chronic mucocuneous candidiasis, and chronic granulomatous disease. While genetic analysis is the definitive approach to establish specific diagnoses of PIDs, flow cytometry provides a tool to effectively evaluate patients with PIDs at relatively low cost.

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8; FHL, familial hemophagocytic lymphohistiocytosis; FOXP3, forkhead box P3; HIES, hyper IgE syndrome; HIGM, hyper IgM syndrome; ICOS, inducible co-stimulator; IFN, interferon; iNKT, invariant natural killer T; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance syndrome; IRAK4, IL-1 receptor-associated kinase 4; JAK3, Janus kinase 3; LAD, leukocyte adhesion deficiency; LPS, lipopolysaccharide; LRBA, lipopolysaccharide-responsive and beige-like anchor protein; mAb, monoclonal antibody; MSMD, Mendelian susceptibility to mycobacterial disease; MyD88, myeloid differentiation primary response gene 88; NOD, nucleotide-binding and oligomerization domain; PBMCs, peripheral blood mononuclear cells; PIDs, primary immunodeficiency diseases; PNP, purine nucleoside phosphorylase; RAG, recombination activating gene; SAP, SLAM-associated protein; SCID, severe combined immunodeficiency; STAT, signal transducer and activator of transcription; TCR, T-cell receptor; Th, T helper; TLR, Toll-like receptor; TNF, tumor necrosis factor; WAS, Wiskott-Aldrich syndrome; WASp, WAS protein; XIAP, X-linked inhibitor of apoptosis; XLA, X-linked agammaglobulinemia; XLP, X-linked lymphoproliferative syndrome; XLT, X-linked thrombocytopenia; X-SCID, X-linked severe combined immunodeficiency; ZAP70, ζ-chain-associated protein kinase of 70 kDa

Introduction

Primary immunodeficiency diseases (PIDs) are a heterogenous group of monogenetic disorders of the immune system, resulting in recurrent and/or severe infections, autoimmunity, auto-inflammation, or malignancies. A careful history focused on the types of infectious agents and other complications are important clues to suspect PID. Laboratory investigations including complete blood count, immunoglobulin levels, antibody titers, assessment of neutrophil function and complement components are also important tools to confirm the diagnosis of PID. As the spectrum of PIDs is expanding, it is often difficult to diagnose PIDs based on clinical and conventional laboratory findings alone. The more recently available genetic investigation is a definitive tool for diagnosing PIDs; however, DNA analysis takes time and is expensive. In contrast, technologies that use physical and chemical characteristics of fluorescent-labeled particles in fluid phase passed through lasers are cheeper than gene analysis, although they need experienced and skilled investigators. Thus, flow cytometry may serve as a bridge between conventional immunological testing and DNA sequencing, offering rapid and accurate results based on single cell analysis.[1]

Application of flow cytometry in the diagnosis of primary immunodeficiency diseases

Flow cytometry is a highly sensitive tool for evaluating the immune system and supporting the diagnosis of PID. The applications of flow cytometry in the evaluation of PIDs are multiplex and include the investigation of specific cell populations and subpopulations, specific cell membrane, intracellular and intranuclear proteins, biologic effects associated with immune defects, and functional immune abnormalities (Table 1).[2]

Quantitative assessment of cell populations and subpopulations is useful for the diagnosis of X-linked agammaglobulinemia (XLA) characterized by the absence of B cells in the peripheral blood. Patients with severe combined immunodeficiency (SCID) lack T cells, while the impact on B and NK cells is variable depending on the genetic defect. Patients with X-linked lymphoproliferative syndrome type 1 (XLP1) have a marked decrease in invariant natural killer T (iNKT) cells. Autoimmune lymphoproliferative syndrome (ALPS) is characterized by increased T-cell receptor (TCR)-α/β-positive double-negative T (DNT) cells. Patients with autosomal dominant hyper IgE syndrome (HIES), and those with chronic mucocutaneous candidiasis (CMCD) present with decreased number of circulating T helper (Th)17 cells.

As specific cell surface proteins are concerned, unique subsets of patients with common variable immunodeficiency (CVID) can be characterized by assessing $CD19^+$ B cells, B-cell activating factor receptor (BAFF-R) on B cells, and the inducible co-stimulator (ICOS) on activated T cells. Patients with X-linked hyper IgM syndrome (X-HIGM) fail to express CD40 ligand (CD40L) on activated T cells, and a group of patients with autosomal recessive hyper IgM syndrome lack CD40 expression on B cells. Mendelian susceptibility to mycobacterial disease (MSMD) has been associated with aberrant interferon (IFN)-γR1 expression on monocytes or deficient IL-12Rβ1 expression on activated T cells. Patients with leukocyte adhesion deficiency type 1 (LAD1) can be identified by absent expression of CD18 on granulocytes. Lymphocytes from patients with X-linked SCID (X-SCID) show deficient CD132 (common γ chain) expression. Patients suffering from gp91-phox- and p22-phox-deficient chronic granulomatous disease (CGD), lacking the

Table 1
Application of flow cytometry in the diagnosis of primary immunodeficiency diseases.

Disease	Test
Evaluate for specific cell population and subpopulation	
XLA	Absent B cells
SCID	Absent T cells and variable number of B and NK cells (depending on defect)
XLP1	Markedly reduced iNKT cells
ALPS	Increased TCR-$\alpha/\beta+$ double-negative ($CD4^-CD8^-$) T cells
HIES and CMCD	Decreased Th17 cells
Evaluate of specific cell surface protein	
CVID	CD19 on B cells
	BAFF-R on B cells
	ICOS on activated T cells
X-linked HIGM	CD40L on activated T cells
Autosomal recessive HIGM	CD40 on B cells
MSMD	IFN-γR1 on monocytes
	IL-12Rβ1 on activated T cells
LAD1	CD18 on granulocytes
X-SCID	CD132 on lymphocytes
gp91-phox and p22-phox deficient CGD	Cytochrome b558 on granulocytes and B cells
IL-17RA deficiency	IL-17RA on lymphocytes and monocytes
Evaluate of specific intracellular protein	
XLA	BTK in monocytes and platelets
Wiskott-Aldrich syndrome and X-linked thrombocytopenia	WASp in lymphocytes and myeloid cells
XLP1	SAP in $CD8^+$ T cells and NK cells
XLP2	XIAP in lymphocytes
FHL2	Perforin in $CD8^+$ T cells and NK cells
FHL3	Munc13-4 in platelets
ZAP70 deficiency	ZAP70 in T cells
p47-phox and p67-phox deficient CGD	p47-phox and p67-phox protein in granulocytes
DOCK8 deficiency	DOCK8 in lymphocytes
CTLA4 haploinsufficiency and LRBA deficiency	CTLA4 in $CD4^+FOXP3^+$ T cells
Evaluate of specific nuclear protein	
IPEX	FOXP3 in $CD4^+CD25^+$ T cells
Evaluate biologic effects	
CVID	Decreased switched memory B cells
Omenn syndrome and hypomorphic SCID	Oligoclonal TCR/diversity
X-linked HIGM	Decreased memory B cells and memory $CD4^+$ T cells
Evaluate function	
MSMD	STAT1 phosphorylation in monocytes in response to IFN-γ
CMCD (STAT1 gain-of-function)	STAT1 phosphorylation in monocytes in response to IFN-γ
FHL3/4/5, Chédiak-Higashi syndrome and Griscelli syndrome	CD107a expression in NK cells and CTLs
XLP2	TNF-α in monocytes in response to muramyl dipeptides
CGD	DHR123 assay in granulocytes, monocytes and B cells
X-SCID and JAK3-deficient SCID	STAT phosphorylation in lymphocytes in response to cytokine stimulation
X-linked HIGM	No binding of CD40L and CD40-Ig
IRAK4 and MyD88 deficiency	TNF-α in monocytes in response to LPS
IL-10R deficiency	STAT3 phosphorylation in lymphocytes in response to IL-10
Infantile-onset multisystem autoimmune disease 1 (heterozygous GOF mutation in STAT 3)	Increased STAT3 phosphorylation

XLA, X-linked agammaglobulinemia; SCID, severe combined immunodeficiency; XLP, X-linked lymphoproliferative syndrome; iNKT, invariant natural killer T; ALPS, autoimmune lymphoproliferative syndrome; TCR, T-cell receptor; HIES, hyper IgE syndrome; CMCD, chronic mucocutaneous candidiasis disease; CVID, common variable immunodeficiency; BAFF-R, B-cell activating factor receptor; ICOS, inducible co-stimulator; HIGM, hyper IgM syndrome; CD40L, CD40 ligand; MSMD, Mendelian susceptibility to mycobacterial disease; IFN, interferon; IL, interleukin; LAD, leukocyte adhesion deficiency; CGD, chronic granulomatous disease; BTK, Bruton tyrosine kinase; WASp, Wiskott-Aldrich syndrome protein; SAP, signaling activation molecule-associated protein; XIAP, X-linked inhibitor of apoptosis; FHL, familial hemophagocytic lymphohistiocytosis; FOXP3, forkhead box P3; ZAP70, ζ-chain -associated protein kinase of 70 kDa; DOCK8, dedicator of cytokinesis 8; CTLA4, cytotoxic T-lymphocyte-associated protein 4; LRBA, LPS responsive beige-like anchor protein; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; STAT, signal transducer and activator of transcription; CTLs, cytotoxic T lymphocytes; DHR, dehydrorhodamine; JAK3, Janus kinase 3; IRAK4, interleukin-1 receptor-associated kinase 4; MyD88, myeloid differentiation primary response gene 88; TNF, tumor necrosis factor; LPS, lypopolysaccharide; GOF, gain-of-function. This table is modified from Reference[2] with permission.

membrane bound cytochrome b558, can be identified using monoclonal antibody (mAb) 7D5 against cytochrome b558 expressed by granulocytes and B cells. Lack of IL-17RA expression on lymphocytes and monocytes is typical for patients with chronic mucocutaneous candida infection and IL-17RA deficiency.

A large number of PIDs can be diagnosed by analyzing expression of specific intracellular proteins. Patients with XLA generally lack Bruton's tyrosine kinase (BTK) expression in monocytes and platelets. Patients suffering from the Wiskott-Aldrich syndrome (WAS) or X-linked thrombocytopenia (XLT) show absent or reduced expression of WAS protein (WASp) in lymphocytes and myeloid cells. Patients with XLP1 lack expression of SLAM-associated protein (SAP) in lymphocytes. Lymphocytes from patients with XLP2 lack expression of X-linked inhibitor of apoptosis (XIAP) protein. Patients experiencing familial hemophagocytic lymphohistiocytosis type 2 (FHL2) can be identified by absent perforin expression in $CD8^+$ T cells and NK cells. Patients with FHL3 demonstrate reduced expression of Munc13-4 in platelets. Patients with ζ-chain-associated protein kinase of 70 kDa (ZAP70) deficiency lack expression of ZAP70 in lymphocytes. Neutrophils from patients with X-linked CGD lack gp91-phox, and those from patients with autosomal recessive CGD due to p47-phox and p67-phox mutation lack relevant protein expression. Most patients with dedicator of cytokinesis 8 (DOCK8) deficiency lack expression of DOCK8 in lymphocytes. Both cytotoxic T-lymphocyte-associated protein 4 (CTLA4) haploinsufficiency and lipopolysaccharide-responsive and beige-like anchor protein (LRBA) deficiency have in common low CTLA4 expression in $CD4^+FOXP3^+$ regulatory T cells. Most patients

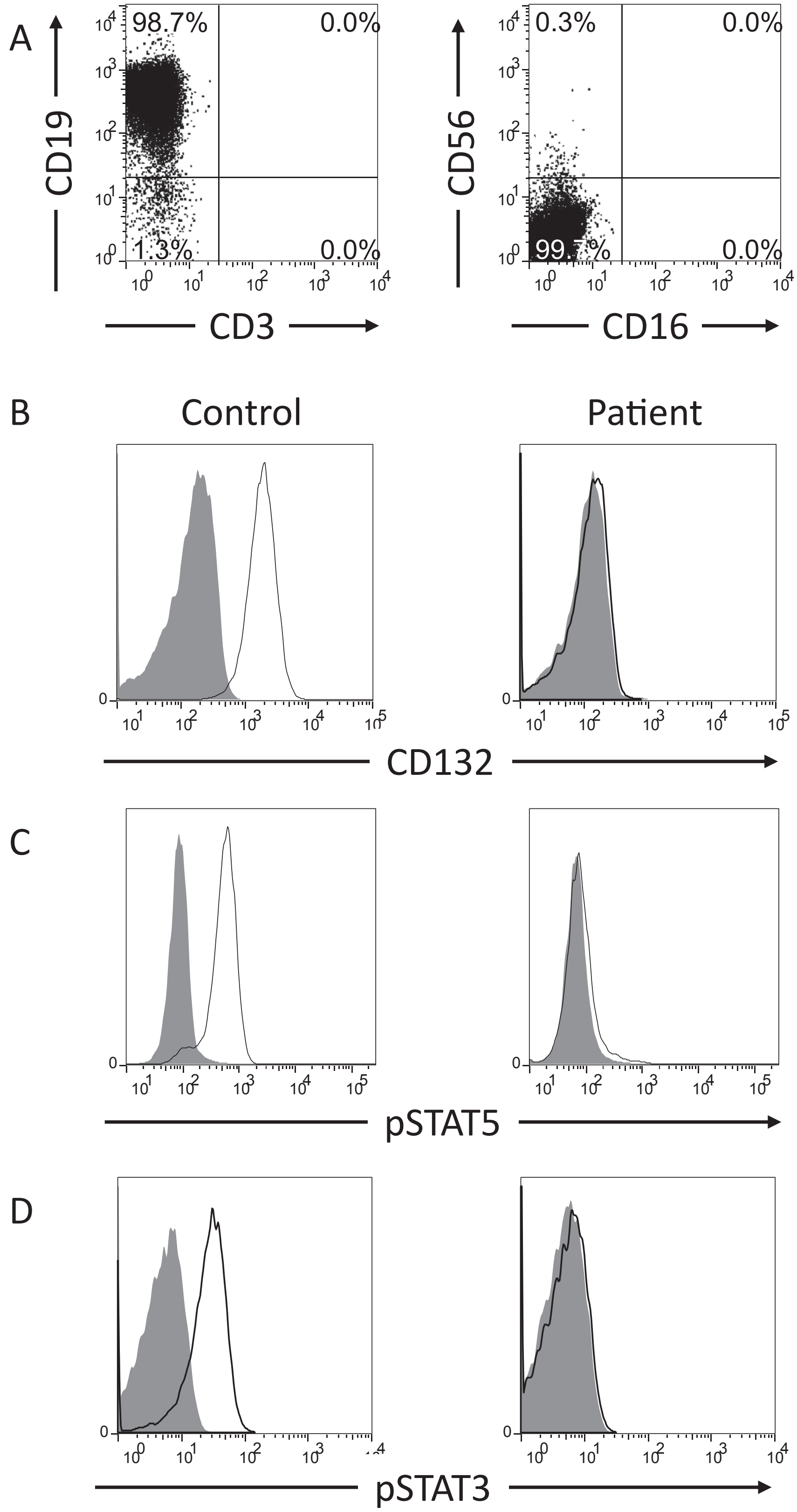

Fig. 1. Flow cytometry in a patient with X-linked severe combined immunodeficiency. **A**, Patient T cells (CD3$^+$), B cells (CD19$^+$), and NK cells (CD16$^+$CD56$^+$) were separated based on the lymphocyte gate. T and NK cells are absent, and the number of B cells are increased. **B**, CD132 (common γ chain) expression by CD19$^+$ B cells. Patient cells lack CD132 expression. Gray shaded area, isotype control; black line, anti-CD132 staining. **C**, Flow cytometric evaluation of STAT5 phosphorylation following IL-2 stimulation. Patient cells fail to respond. Gray shaded area, no stimulant; black line, 20 min post-IL-2 stimulation. **D**, Flow cytometric evaluation of STAT3 phosphorylation following IL-21 stimulation. Patient cells fail to respond. Gray shaded area, no stimulant; black line, 20 min post-IL-21 stimulation.

presenting with immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance syndrome (IPEX) have low or absent nuclear forkhead box P3 (FOXP3) expression by $CD4^+CD25^+$ regulatory T cells.

Flow cytometry techniques are now available to evaluate biologic effects of mutated genes, and the results can be used for the diagnosis of relevant PIDs. Most patients with CVID or hyper IgM syndromes have decreased switched memory B cells. Omenn syndrome and most hypomorphic SCID syndromes are characterized by an oligoclonal T-cell repertoire.

The introduction of functional tests using flow cytometry has provided a powerful tool to evaluate important pathways of cognate and innate immunity. Patients with MSMD due to mutations in genes of the IL12/23-IFNγ pathway can be identified by demonstrating reduced phosphorylation of signal transducer and activator of transcription (STAT)1 expression in response to stimulation with cytokines. Peripheral blood mononuclear cells (PBMCs) from patients with CMCD respond with reduced phosphorylation of STAT1 when stimulated with IFN-γ. Patients with FHL3/4/5, Chédiak-Higashi and Gricelli syndrome demonstrate reduced CD107a expression in resting NK cells and in cytotoxic T lymphocytes (CTLs). Monocytes of patients with XLP2 respond with reduced tumor necrosis factor (TNF)-α production by monocytes in response to muramyl dipeptide. CGD patients regardless of the molecular defect show reduced or absent dihydrorhodamine (DHR) 123 reduction in granulocytes, monocytes and B cells. Patients with X-SCID and Janus kinase 3 (JAK3)-deficient SCID demonstrate reduced phosphorylation of STAT3 and STAT5 in response to cytokine-stimulation. IL-1 receptor-associated kinase 4 (IRAK4) and myeloid differentiation primary response gene 88 (MyD88) deficiencies can be identified by reduced TNF-α production in monocytes in response to lipopolysaccharide (LPS). Patients with IL-10 receptor deficiency demonstrate reduced STAT3 phosphorylation when stimulated with IL-10. Infantile-onset multisystem autoimmune disease 1, caused by heterozygous gain-of-function mutation in STAT3, is typically associated with increased STAT3 phosphorylation by unstimulated lymphocytes.

The following are examples illustrating the application of flow cytometry for the diagnosis of molecularly defined PIDs.

Severe combined immunodeficiency

SCID disorders, the most severe forms of PID, are generally characterized by complete absence of T-cell mediated immunity and impaired B-cell function.[3] Patients with SCID can be classified

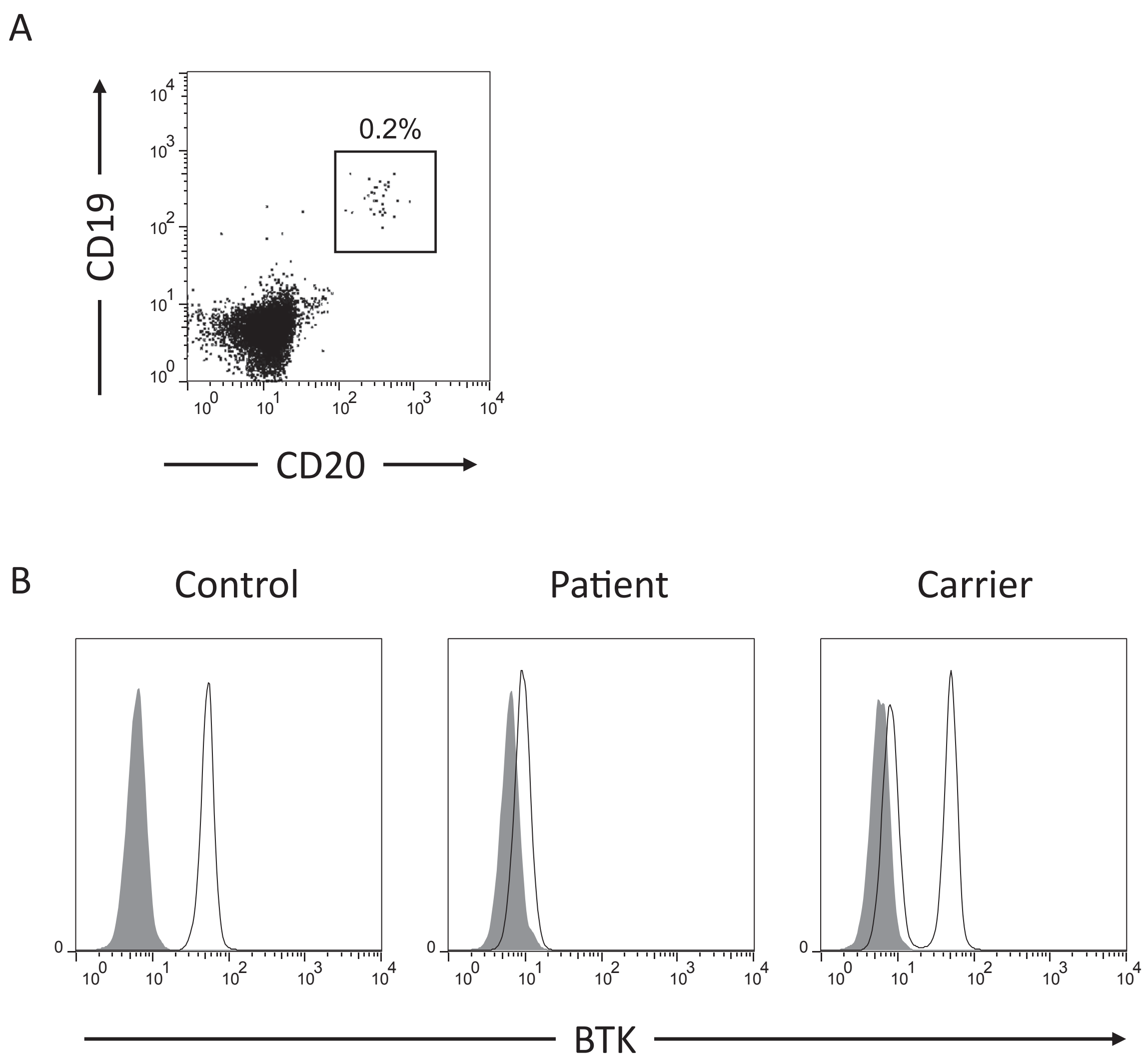

Fig. 2. Flow cytometry in a patient with X-linked agammaglobulinemia (XLA). **A**, $CD19^+CD20^+$ B cells are markedly reduced. **B**, Bruton's tyrosine kinase (BTK) expression is absent in patient monocytes. A bimodal or mosaic pattern of BTK expression is demonstrated in the heterozygous carrier. Gray shaded area, isotype control; black line, anti-BTK monoclonal antibody.

by immunophenotypic characteristics based on flow cytometry. $T^-B^-NK^-$ SCID includes reticular dysgenesis and adenosine deaminase (ADA) deficiency. $T^-B^-NK^+$ SCID suggest mutations affecting the recombination activating genes (*RAG)1* and *RAG2*, Artemis, DNA ligase IV, and Cernunnos. $T^-B^+NK^-$ phenotype is characteristic for X-SCID and JAK3 deficiency. $T^-B^+NK^+$ SCID includes IL-7Rα, CD3δ, CD3ε and CD3ζ deficiencies. In X-SCID, representing close to half of all SCID patients,[4] T and NK cells are absent while B cell counts are normal or high (Fig. 1A). The gene responsible for X-SCID is *IL2RG*, coding for the common γ chain (CD132). Therefore, the absence of CD132 by flow cytometry strongly suggests X-SCID (Fig. 1B), although a few patients with mutations in the cytoplasmic domain of the *IL2RG* may express normal CD132. Since the common γ chain is also part of the IL-4, IL-7, IL-9, IL-15, and IL-21 receptors, these cytokines constitute the specific ligands for all pathways that depend on a functional common γ chain. Phosphorylation of intracellular STATs can be evaluated by flow cytometry using mAbs that recognize only STATs that are phosphorylated. As shown in Figure 1C,D, patients with X-SCID have impaired tyrosine phosphorylation of STAT5 and STAT3 in response to stimulation with IL-2 and IL-21, respectively. This assay also identifies JAK3 deficiency because JAK3 interacts intracellularly with the common γ chain. ZAP70 deficiency is a form of SCID characterized by CD8 deficiency; patients with ZAP70 deficiency lack expression of ZAP70 in T cells.[5]

X-linked agammaglobulinemia

XLA is characterized by the absence of circulating B cells and severe reduction of all serum immunoglobulins due to mutations in the *BTK* gene. Absent or markedly reduced B cell numbers, determined by flow cytometry based on the lack of CD19- and/or CD20-expressing cells, are typical for all forms of agammaglobulinemia (Fig. 2A). Therefore, assessment of BTK is mandatory for the diagnosis of XLA.[6] Because patients with XLA have no B cells, intracellular BTK expression has to be evaluated in monocytes or platelets (Fig. 2B).[7,8] This technique can also be used for the detection of XLA carriers. Absent of reduced expression of BTK strongly suggests XLA, but normal quantity of BTK does not rule out the diagnosis, as some patients with XLA express normal amount of nonfunctional BTK protein.[9] B cell-deficient patients with wild type *BTK* may have autosomal recessive agammaglobulinemia, and sequence analysis of μ heavy chain, Igα, Igβ, λ5, BLNK, E47 or PI3KR1 is required.

Hyper IgM syndromes

HIGM syndromes are a group of genetic disorders affecting molecules involved in B cell class switch recombination and

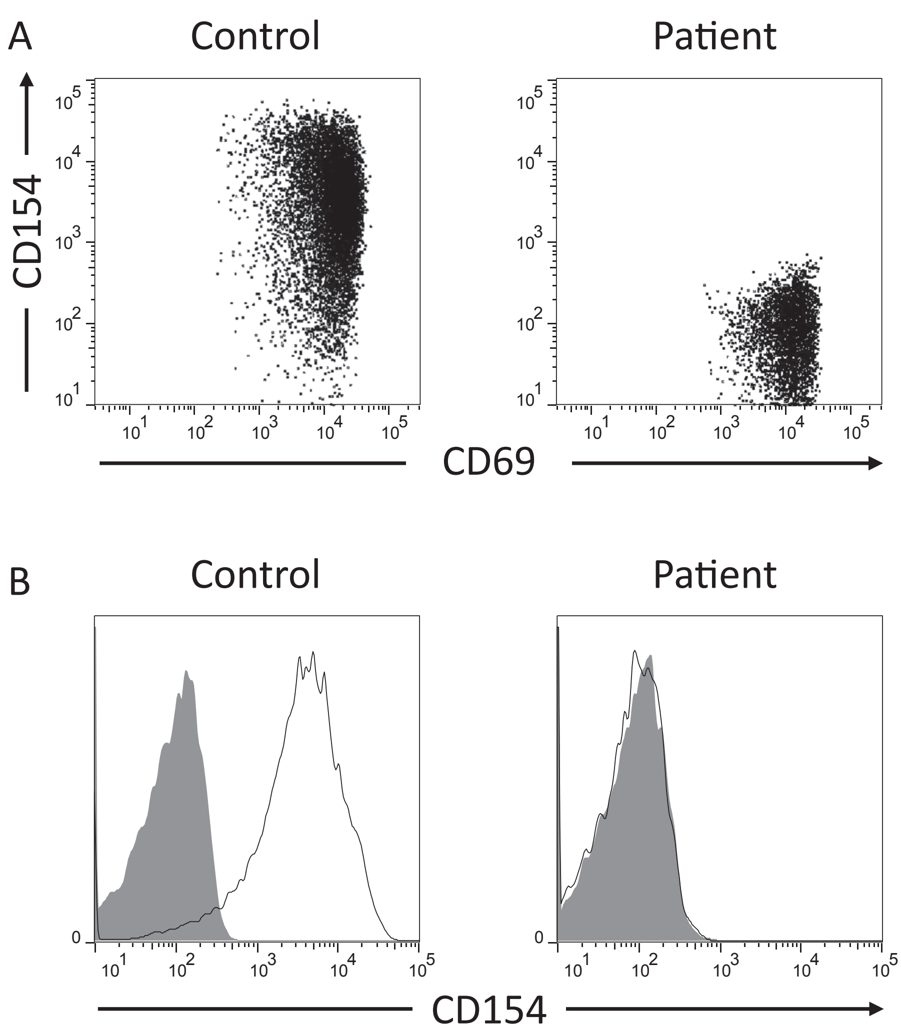

Fig. 3. Flow cytometric identification of CD154 (CD40L) on activated T cells in a patient with X-linked hyper IgM syndrome. **A**, Peripheral blood mononuclear cells were cultured with phorbol myristate acetate and ionomycin for 3.5 h. The cells are CD69-positive, indicating activated T cells, but only control CD69-positive cells express CD152. **B**, CD154 expression on $CD3^+CD8^-$ T cells was evaluated. The patient's cells failed to express CD154. Gray shaded area, isotype control; black line, anti-CD154 monoclonal antibody.

somatic hypermutation.[10] Affected patients present with normal or increased serum IgM, and low levels of IgG and IgA. While mutations in several genes have been associated with HIGM, the most frequently affected gene is *CD40L* with X-linked recessive inheritance. CD40L-deficient patients develop not only bacterial but also opportunistic infections and malignancies, implying the important role of CD40L in T cell function. CD40L (CD154) expression by activated CD4$^+$ T cells is absent or reduced when assessed by anti-CD40L specific mAbs in most but not all patients with X-linked HIGM (Fig. 3), since some mutations result in non-functional protein that nevertheless can bind to epitope neutral mAbs.[11] In contrast, only functional CD40L can bind to the CD40-Ig construct, providing a flow cytometry-based functional assay for X-linked HIGM.[12] CD40 deficiency, one of several autosomal recessive HIGM syndromes, is a phenocopy of CD40L deficiency that can be identified by assessing CD40 expression on B cells, monocytes, or dendritic cells.[13] Except for CD40L and CD40 deficiency, none of the other HIGM syndrome can be identified by flow cytometry.

Common variable immunodeficiency

CVID is a heterogenous group of disorders characterized by hypogammaglobulinemia, defective specific antibody production and increased susceptibility to recurrent and chronic infections, and often to autoimmunity, lymphoproliferative disorders and cancer.[14] Patients with CVID have normal or low numbers of B cells. B cells can be subdivided into naïve (CD27$^-$IgD$^+$IgM$^+$), IgM memory (CD27$^+$IgD$^+$IgM$^+$), and switched memory (CD27$^+$IgD$^-$IgM$^-$) B cells based on CD27 and IgD/IgM expression.[15] Most patients with CVID show a decreased number of switched memory B cells. Decreased numbers of switched memory B cells are also observed in HIGM syndromes.[16]

A small subset of CVID are caused by mutations in *ICOS*, *CD19*, and *BAFFR* (*TNFRSF13C*). These patients can be screened by flow cytometry. Patients with ICOS deficiency were reported to have reduced up-regulation of ICOS by activated T cells.[17] BAFFR is constitutively expressed on B cells, and patients with *BAFFR* mutations show reduced expression of this protein.[18] CD19 forms complexes with CD21, CD81, and CD225 which collaborate with the B cell receptor upon antigen recognition. Absence of CD19 expression on B cells has been observed in patients with CD19 and CD81 deficiencies.[19–21]

Wiskott-Aldrich syndrome and X-linked thrombocytopenia

WAS is a rare X-linked disorder characterized by persistent microthrombocytopenia, eczema, cellular and humoral immunodeficiency, and an increased risk of autoimmune disease and hematologic malignancy.[22] WAS is caused by mutations in the *WAS* gene encoding the WASp; this gene is also responsible for XLT and X-linked neutropenia. Monoclonal antibodies against WASp are useful for screening patients suspected to have WAS or XLT (Fig. 4).[23,24] This technique is also of value in the evaluation of chimerism after hematopoietic stem cell transplantation and somatic reversion mosaicism of the *WAS* gene.[25,26]

X-linked lymphoproliferative syndrome

XLP, a rare PID with susceptibility to Epstein–Barr virus infection, is clinically characterized by hemophagocytic lymphohistiocytosis and hypogammaglobulinemia, with or without lymphoma. XLP is classified into type 1 (XLP1) caused by mutations in the *SH2D1A* gene encoding SAP and type 2 (XLP2) caused by mutations in the *XIAP* or *BIRC4* gene encoding XIAP. Flow cytometric detection of intracellular SAP and XIAP proteins are useful screening tests for the identification of patients with XLP1 and XLP2, respectively

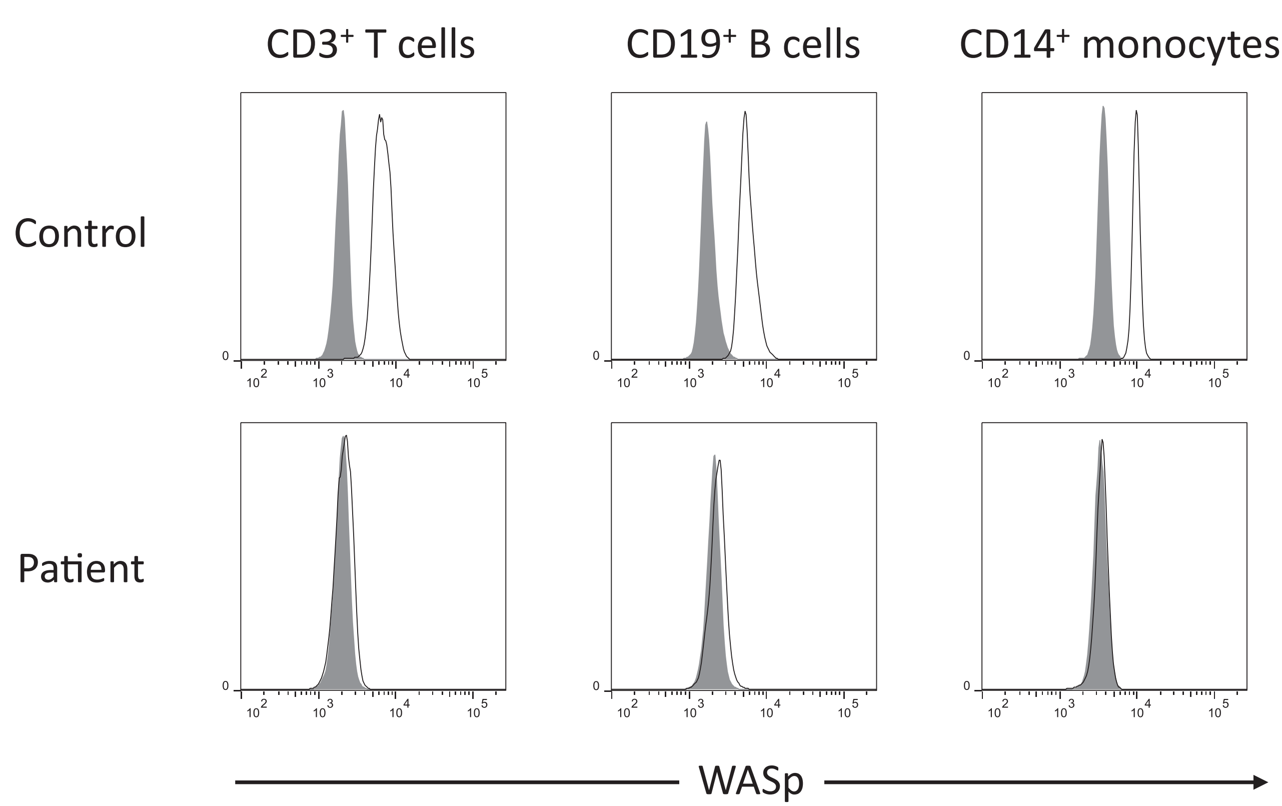

Fig. 4. Flow cytometric detection of WASp in a patient with Wiskott-Aldrich syndrome. Cytoplasmic WASp expression was markedly reduced in patient CD3$^+$ T cells, CD19$^+$ B cells, and CD14$^+$ monocytes. Gray shaded area, isotype control; black line, anti-WASp mAb.

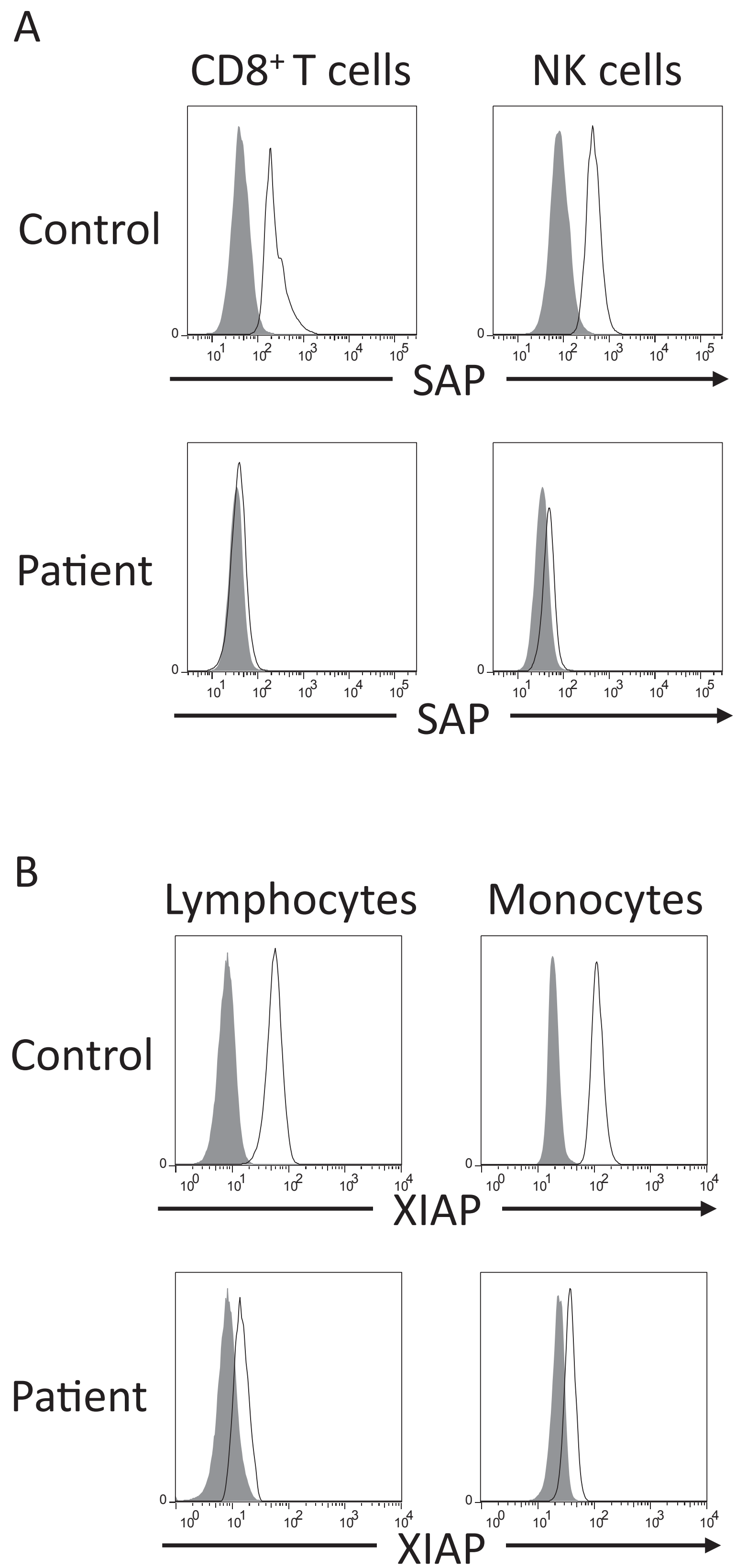

Fig. 5. Flow cytometry in patients with X-linked lymphoproliferative syndrome type 1 (XLP1) and XLP2. **A**, SAP expression was markedly reduced in $CD8^+$ T cells and $CD56^+$ NK cells from a patient with XLP1. **B**, XIAP expression was reduced in lymphocytes and monocytes from a patient with XLP2. Gray shaded area, isotype control; black line, anti-SAP or anti-XIAP monoclonal antibody.

(Fig. 5A,B).[27,28] While patients with XLP1 have extremely reduced numbers of iNKT cells, patients with XLP2 have variable low numbers of iNKT cells. Flow cytometry is also useful to identify atypical cases of XLP including somatic reversion mosaicism of XLP1, and female XLP2.[29,30] Because XIAP plays an essential role in nucleotide-binding oligomerization domain protein (NOD)1/2 signaling, flow cytometric assessment of TNF-α production by monocytes in response to NOD2 stimulation by muramyl dipeptide is a useful functional approach for diagnosing XIAP deficiency. TNF-α production was found to be severely diminished in all patients with XIAP deficiency studied.[31]

Familial hemophagocytic lymphohistiocytosis

FHL is a group of genetically determined, life-threatening disorders associated with the uncontrolled proliferation of activated lymphocytes and histiocytes secreting large amounts of inflammatory cytokines.[32] Genetic defects affecting granule-mediated cytotoxicity are associated with FHL, including perforin (FHL2), Munc13-4 (FHL3), syntaxin 11 (FHL4), and Munc 18-2 (FHL5) deficiencies. FHL2 can be evaluated by assessing perforin expression in CD56$^+$CD16$^+$ NK cells and CD8$^+$ T cells (Fig. 6A),[33] and FHL3 can be screened by detecting Munc13-4 expression in platelets (Fig. 6B).[34] Assessment of the release of cytolytic granules by measuring surface expression of CD107a by NK or cytotoxic T cells is useful for the diagnosis of FHL3.[35,36] This assay has also been suggested for the diagnosis of FHL 4, FHL5, Chédiak-Higashi syndrome and Griscelli syndrome, all having in common defects in granule-mediated cytotoxic pathways.[37]

Autoimmune lymphoproliferative syndrome

ALPS is a disorder of lymphocyte homeostasis characterized by chronic non-malignant lymphoproliferation, autoimmune manifestations (mainly autoimmune cytopenia) and an increased incidence of lymphoid malignancies.[38] Most patients with ALPS harbor mutations in genes which regulate the extrinsic Fas-mediated programmed cell death pathway (*FAS*, *FASLG* and *CASP10*). An immunological hallmark of this syndrome is the increased level of circulating TCR-α/β^+CD4$^-$CD8$^-$ T cells, referred to as DNT cells (Supplementary Fig. 1A). While control T cells undergo robust apoptosis as shown by annexin V-positive cells following stimulation with anti-FAS antibody, ALPS patient T cells do not (Supplementary Fig. 1B).

IPEX syndrome

IPEX syndrome, a rare X-linked autoimmune disorder caused by mutations in the *FOXP3* gene, is characterized by severe enteropathy, endocrinopathies (diabetes and/or thyroiditis), and eczematous dermatitis.[39] FOXP3 plays a critical role in the development

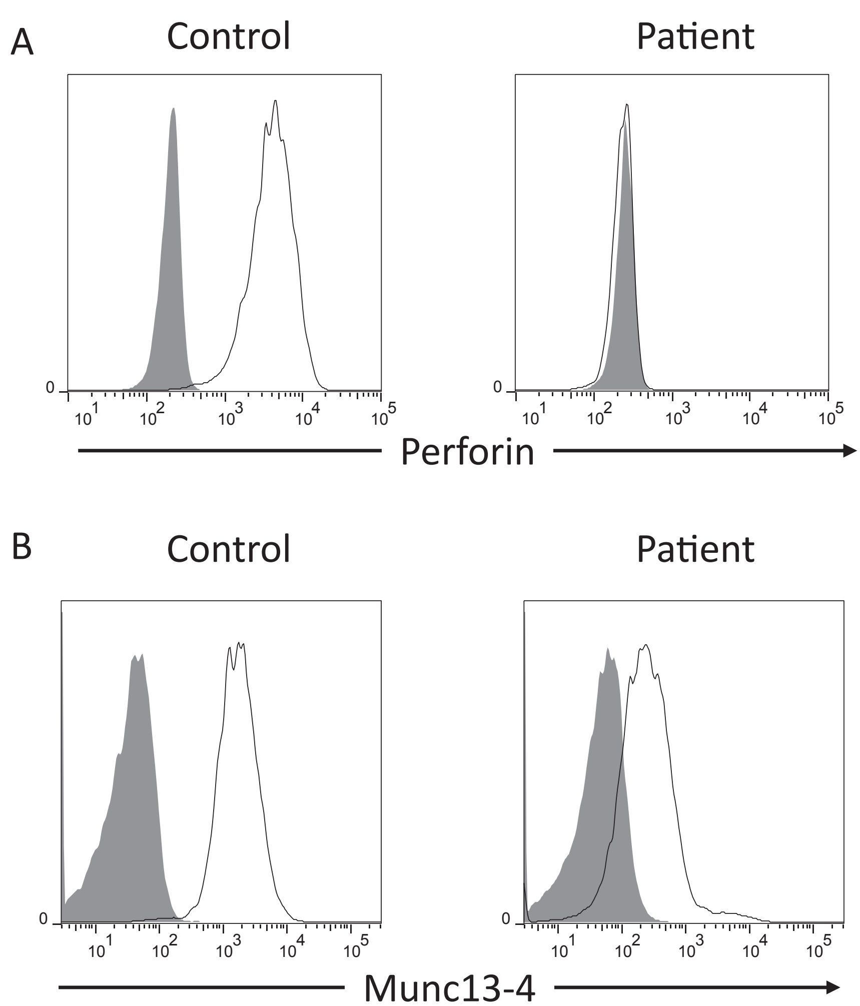

Fig. 6. Flow cytometric detection of patients with FHL2 and FHL3. **A**, Reduced perforin expression in NK cells (CD3$^-$CD56$^+$CD16$^+$ lymphocyte population) from a FHL2 patient. Solid histograms represents staining with isotype control antibody and open histograms represent staining with anti-perforin antibody. **B**, Reduced Munc13-4 expression in platelets (CD41a$^+$ population) from a FHL3 patient. Solid histograms represent staining with control rabbit serum and open histograms represents staining with anti-Munc13-4 rabbit antibody.

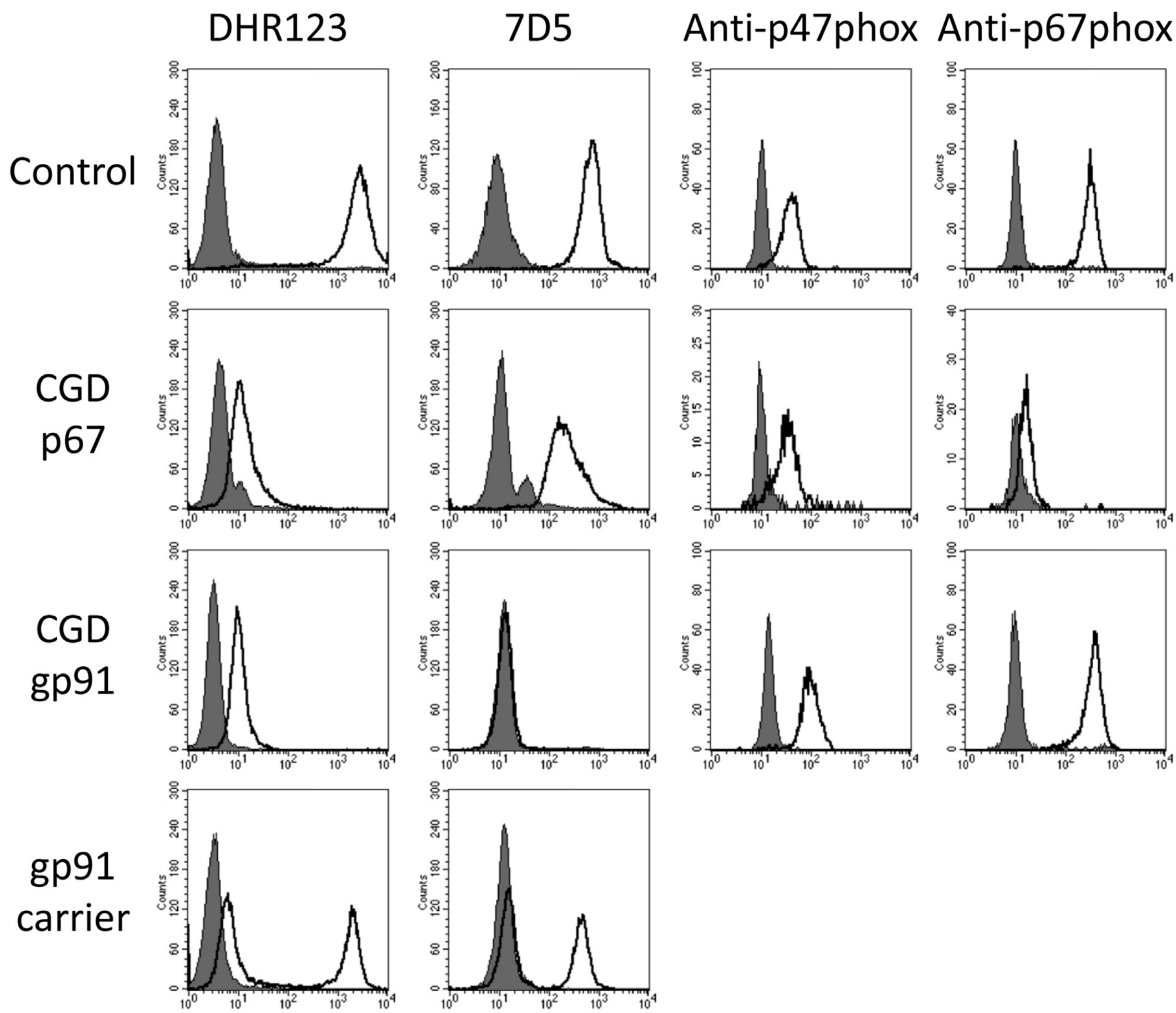

Fig. 7. Flow cytometric analysis of dihydrorhodamine (DHR) oxidation and expression of p47phox, p67phox and gp91phox in CGD patients and a carrier mother. In the DHR assay, granulocytes were analyzed using DHR123 as a fluorescent probe before (solid histogram) and after (open histogram) stimulation with phorbol myristate acetate (first column of panels). Expression of gp91phox in patient granulocytes, as assessed using 7D5 (second column of panels). Expression of p47phox and p67phox in CD14^{+} monocytes are shown in third and fourth column. Solid histograms indicate isotype control antibody and open histograms represent anti-subunit monoclonal antibodies.

and function of CD4^{+}CD25^{+} regulatory T (Treg) cells. Most IPEX patients lack or have reduced numbers of CD4^{+}CD25^{+}FOXP3^{+} or CD4^{+}CD25^{+}CD127^{low} Treg cells. Interestingly, a sufficient number of CD4^{+}CD25^{+}CD127^{low} cells were observed in patients with hypomorphic *FOXP3* mutations.[40] Because CD25 (α chain of the IL-2 receptor) and STAT5b affect Treg development and function, patients with CD25 or STAT5b mutations present with an IPEX-like phenotype.[41,42] Although reduced in number, Treg cells from these patients express FOXP3.

CTLA4 haploinsufficiency and LRBA deficiency

CTLA4 is a costimulatory molecule expressed by activated T cells, and, similar to the T-cell costimulatory molecule CD28, binds to B7-1 (CD80) and B7-2 (CD86) on the surface of antigen-presenting cells. CTLA4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal. Heterozygous loss-of-function mutations in the *CTLA4* gene have been identified in CVID patients with IPEX-like phenotype including enteropathy and autoimmune cytopenias.[43,44] Patients with CTLA4 haploinsufficiency show reduced expression of CTLA4 in activated CD4^{+}FOXP3^{+} T cells (Supplementary Fig. 2). Biallelic loss-of-function mutations in *LRBA* were identified in patients with the clinical diagnosis of CVID with ALPS- or IPEX-like manifestations.[45,46] A common denominator is early onset of severe autoimmunity, often associated with recurrent infections and lymphoproliferative disease with increased risk of lymphoma. Because LRBA colocalizes with CTLA4 in endosomal vesicles, LRBA deficiency increases CTLA4 turnover, resulting in reduced levels of CTLA4 protein in FOXP3^{+} regulatory and activated conventional T cells.[47] The phenotypic similarity between LRBA and CTLA4 deficiencies may be explained by this common defect in CTLA4 expression.

IRAK4 and MyD88 deficiency

IRAK4 is a kinase that plays a crucial role in Toll-like receptor (TLR) and IL-1 receptor signaling. Ligand binding to these receptors triggers the recruitment of the adaptor proteins MyD88, IRAK4, and IRAK1, resulting in downstream signal transduction. Autosomal recessive IRAK4 and MyD88 deficiency impair TLR and IL-1 receptor-mediated immunity, resulting in invasive bacterial, especially pneumococcal infections.[48] Intracellular TNF-α production in monocytes in response to LPS (a TLR4 ligand) was assayed by flow cytometry and found to be diminished in patients with IRAK4 deficiency (Supplementary Fig. 3).[49] This assay may also be useful for the screening of patients with MyD88 deficiency.

Mendelian susceptibility to mycobacterial disease

MSMD represents a group of PIDs characterized by vulnerability to infection with weakly virulent mycobacteria and Salmonella.[50] Affected patients may have mutations in genes involved in the IL-12/23-IFN-γ pathway. Patients with IL-12Rβ1 and IFNγR1 deficiencies can be screened by flow cytometry. Most patients with IL-12Rβ1 and autosomal recessive IFNγR1 deficiencies demonstrate absence of cell surface protein. In contrast, IFNγR1 expression was increased in a patient with autosomal dominant form of IFNγR1 deficiency due to overexpression of the abnormal IFNγR1 chain (Supplementary Fig. 4).

Chronic mucocutaneous candidiasis

CMCD is characterized by persistent or recurrent *C. albicans* infections of skin, nail, and mucosal membranes.[51] CMCD refers to a hetergenous group of PIDs including autosomal dominant HIES associated with heterozygous *STAT3* mutation, IL-12p40 deficiency, IL-12Rβ1 deficiency, and autoimmune polyendocrinopathy-candidiasis–ectodermal dystrophy/dysplasia (APECED). Patients with CMCD often have decreased levels of Th17 cells, as do patients with HIES.[52] Those with APECED may develop neutralizing autoantibodies against IL-17A, IL-17F, and/or IL-22, explaining the development of CMCD. Of the recently discovered autosomal recessive IL-17RA and autosomal dominant IL-17F deficiencies associated with CMCD,[53] IL-17RA deficiency can be readily diagnosed by the absence of IL-17RA on the surface of circulating lymphocytes and monocytes.[54] The most common cause of CMCD are heterozygous gain-of-function mutations in *STAT1*.[55,56] Flow analysis readily recognizes elevated levels of phosphorylated STAT1 that persist following IFN-γ stimulation of monocytes from patients with *STAT1* gain-of-function mutations (Supplementary Fig. 5).[57] In contrast to control cells, the tyrosine kinase inhibitor, staurosporine, did not reduce STAT1 phosphorylation in monocytes obtained from patients with STAT1 gain-of-function mutations.

Chronic granulomatous disease

CGD is a genetically heterogeneous PID affecting bactericidal function of phagocytes and characterized by recurrent bacterial and fungal infections. CGD is caused by defects in the NADPH oxidase complex, which is responsible for the phagocyte respiratory burst leading to the generation of superoxide and other reactive oxygen species. Mutations in five components ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) of the NADPH complex account for the X-linked and autosomal recessive forms of CGD. Laboratory diagnosis of CGD is performed by the measurement of superoxide production, and is evaluated by flow cytometry using DHR123 oxidation (Fig. 7). This method allows the distinction between X-linked and autosomal recessive CGD, and detection of carriers of X-linked CGD.[58] Subtypes of CGD can be determined by genetic as well as flow cytometric analysis. The 7D5 mAb recognizes the surface components of NADPH oxidase, and patients with $gp91^{phox}$ and $p22^{phox}$ deficiencies can be diagnosed using this antibody (Fig. 7).[59] Patients with $p47^{phox}$ and $p67^{phox}$ deficiencies can be identified with intracellular staining using anti-$p47^{phox}$ and $p67^{phox}$ specific monoclonal antibodies, respectively.[60]

All the staining protocols are available in Supplementary Methods.

Conclusion

Flow cytometry is an instrumental tool for the evaluation and diagnosis of PIDs. The use of flow cytometry provides rapid results and often suggests the correct diagnosis. While genetic analysis delivers a definitive diagnosis, flow cytometry plays an important role in the cost-effective evaluation of patients suspected to have PID.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.alit.2017.06.003

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

HK designed the study and wrote the manuscript. AH, TO, TYa, TW, TH and SO performed the flow cytometry and wrote the draft. MY and TYe performed the flow cytometric analysis and collected the data. RN, MT, KI, HDO and TM contributed to critical discussion. All authors read and approved the final manuscript.

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